SECTION 7

DILUTION WATER

7.1 TYPES OF DILUTION WATER

7.1.1 The type of dilution water used in effluent toxicity tests will depend largely on the objectives of the study:

7.1.1.1 If the objective of the test is to estimate the absolute acute toxicity of the effluent, a synthetic (standard) dilution water is used. If the test organisms have been cultured in water which is different from the test dilution water, a second set of controls, using culture water, should be included in the test.

7.1.1.2 If the objective of the test is to estimate the acute toxicity of the effluent in uncontaminated receiving water, the test may be conducted using dilution water consisting of a single grab sample of receiving water (if non-toxic), collected either upstream and outside the influence of the outfall, or with other uncontaminated natural water (ground or surface water) or standard dilution water having approximately the same characteristics (hardness and/or salinity) as the receiving water. Seasonal variations in the quality of surface waters may affect effluent toxicity. Therefore, the hardness of fresh receiving water, and the salinity of saline receiving water samples should be determined before each use. If the test organisms have been cultured in water which is different from the test dilution water, a second set of controls, using culture water, should be included in the test.

7.1.1.3 If the objective of the test is to determine the additive or mitigating effects of the discharge on already contaminated receiving water, the test is performed using dilution water consisting of receiving water collected immediately upstream or outside the influence of the outfall. A second set of controls, using culture water, should be included in the test.

7.1.2 An acceptable dilution water is one which is appropriate for the objectives of the test; supports adequate performance of the test organisms with respect to survival, growth, reproduction, or other responses that may be measured in the test (i.e., consistently meets test acceptability criteria for control responses); is consistent in quality; and does not contain contaminants that could produce toxicity. Receiving waters, synthetic waters, or synthetic waters adjusted to approximate receiving water characteristics may be used for dilution provided that the water meets the above listed qualifications for an acceptable dilution water. USEPA (2000a) provides additional guidance on selecting appropriate dilution waters.

7.1.3 When dual controls (one control using culture water and one control using dilution water) are used (see Subsections 7.1.1.1 - 7.1.1.3 above), the dilution water control should be used to determine test acceptability. It is also the dilution water control that should be compared to effluent treatments in the calculation and reporting of test results. The culture water control should be used to evaluate the appropriateness of the dilution water source. Significant differences between organism responses in culture water and dilution water controls could indicate toxicity in the dilution water and may suggest an alternative dilution water source. USEPA (2000a) provides additional guidance on dual controls.

7.2 STANDARD, SYNTHETIC DILUTION WATER

7.2.1 Standard, synthetic, dilution water is prepared with deionized water and reagent grade chemicals or mineral water (Tables 7 and 8) and commercial sea salts (FORTY FATHOMS®, HW MARINEMIX®) (Table 9). The source water for the deionizer can be groundwater, or tap water.

7.2.2 DEIONIZED WATER USED TO PREPARE STANDARD, SYNTHETIC, DILUTION WATER

7.2.2.1 Deionized water is obtained from a MILLIPORE® MILLI-Q®, MILLIPORE® QPAK™$_2$, or equivalent system. It is advisable to provide a preconditioned (deionized) feed water by using a Culligan®, Continental®, or equivalent, system in front of the MILLIPORE® System to extend the life of the MILLIPORE® cartridges.

7.2.2.2 The recommended order of the cartridges in a four-cartridge deionizer (i.e., MILLI-Q® System or equivalent) is: (1) ion exchange, (2) ion exchange, (3) carbon, and (4) organic cleanup (such as ORGANEX-Q®, or equivalent), followed by a final bacteria filter. The QPAK™$_2$ water system is a sealed system which does not allow for the rearranging of the cartridges. However, the final cartridge is an ORGANEX-Q® filter, followed by a final bacteria filter. Commercial laboratories using this system have not experienced any difficulty in using the water for culturing or testing. Reference to the MILLI-Q® systems throughout the remainder of the manual includes all MILLIPORE® or equivalent systems.

7.2.3 STANDARD, SYNTHETIC FRESHWATER

7.2.3.1 To prepare 20 L of standard, synthetic, moderately hard, reconstituted water, use the reagent grade chemicals in Table 7 as follows:

1. Place 19 L of MILLI-Q®, or equivalent, deionized water in a properly cleaned plastic carboy.
2. Add 1.20 g of $MgSO_4$, 1.92 g $NaHCO_3$, and 0.080g KCl to the carboy.
3. Aerate overnight.
4. Add 1.20 g of $CaSO_4 \bullet 2\ H_2O$ to 1 L of MILLI-Q® or equivalent deionized water in a separate flask. Stir on magnetic stirrer until calcium sulfate is dissolved, add to the 19 L above, and mix well.
5. For *Ceriodaphnia* culture and testing, add sufficient sodium selenate (Na_2SeO_4) to provide 2 µg selenium per liter of final dilution water.
6. Aerate the combined solution vigorously for an additional 24 h to dissolve the added chemicals and stabilize the medium.
7. The measured pH, hardness, etc., should be as listed in Table 7.

7.2.3.2 To prepare 20 L of standard, synthetic, moderately hard, reconstituted water, using 20% mineral water such as PERRIER® Water, or equivalent (Table 8), follow the instructions below.

1. Place 16 L of MILLI-Q® or equivalent deionized water in a properly cleaned plastic carboy.
2. Add 4 L of PERRIER® Water, or equivalent.
3. Aerate vigorously for 24 h to stabilize the medium.
4. The measured pH, hardness, and alkalinity of the aerated water will be as indicated in Table 8.
5. This synthetic water is referred to as diluted mineral water (DMW) in the toxicity test methods.

7.2.4 STANDARD, SYNTHETIC SEAWATER

7.2.4.1 To prepare 20 L of a standard, synthetic, reconstituted seawater (modified GP2), with a salinity of 31‰ (Table 9), follow the instructions below. Other salinities can be prepared by making the appropriate dilutions.

1. Place 20 L of MILLI-Q® or equivalent deionized water in a properly cleaned plastic carboy.
2. Weigh reagent grade salts listed in Table 9 and add, one at a time, to the deionized water. Stir well after adding each salt.
3. Aerate the final solution at a rate of 1 L/h for 24 h.
4. Check the pH and salinity.

Larger or smaller volumes of modified GP2 can be prepared by using proportionately larger or smaller amounts of salts and dilution water.

7.2.4.2 Synthetic seawater can also be prepared by adding commercial sea salts, such as FORTY FATHOMS®, HW MARINEMIX® or equivalent, to deionized water. For example, thirty-one parts per thousand (31‰) FORTY FATHOMS® can be prepared by dissolving 31 g of product per liter of deionized water. The salinity of the resulting solutions should be checked with a refractometer.

7.3 USE OF RECEIVING WATER AS DILUTION WATER

7.3.1 If the objectives of the test require the use of uncontaminated surface water as dilution water, and the receiving water is uncontaminated, it may be possible to collect a sample of the receiving water close to the outfall, but upstream from or beyond the influence of the effluent. However, if the receiving water is contaminated, it may be necessary to collect the sample in an area "remote" from the discharge site, matching as closely as possible the physical and chemical characteristics of the receiving water near the outfall.

7.3.2 The sample should be collected immediately prior to the test, but never more than 96 h before the test begins. Except where it is used within 24 h, or in the case where large volumes are required for flow-through tests, the sample should be chilled to 4°C during or immediately following collection, and maintained at that temperature prior to use in the test.

7.3.3 In the case of freshwaters, the regulatory authority may require that the hardness of the dilution water be comparable to the receiving water at the discharge site. This requirement can be satisfied by collecting an uncontaminated surface water with a suitable hardness, or adjusting the hardness of an otherwise suitable surface water by addition of reagents as indicated in Table 7.

7.3.4 In an estuarine environment, the investigator should collect uncontaminated water having a salinity as near as possible to the salinity of the receiving water at the discharge site. Water should be collected at slack high tide, or within one hour after high tide. If there is reason to suspect contamination of the water in the estuary, it is advisable to collect uncontaminated water from an adjacent estuary. At times it may be necessary to collect water at a location closer to the open sea, where the salinity is relatively high. In such cases, deionized water or uncontaminated freshwater is added to the saline water to dilute it to the required test salinity. Where necessary, the salinity of a surface water can be increased by the addition of artificial sea salts, such as FORTY FATHOMS® or equivalent, a natural seawater of higher salinity, or hypersaline brine. Instructions for the preparation of hypersaline brine by concentrating natural seawater are provided below.

7.3.5 Receiving water containing debris or indigenous organisms, that may be confused with or attack the test organisms, should be filtered through a sieve having 60 µm mesh openings prior to use.

TABLE 7. PREPARATION OF SYNTHETIC FRESHWATER USING REAGENT GRADE CHEMICALS[1]

	Reagent Added (mg/L)[2]				Approximate Final Water Quality		
	$NaHCO_3$	$CaSO_4 \cdot 2H_2O$	$MgSO_4$	KCl	pH[3]	Hardness[4]	Alkalinity[4]
Very soft	12.0	7.5	7.5	0.5	6.4-6.8	10-13	10-13
Soft	48.0	30.0	30.0	2.0	7.2-7.6	40-48	30-35
Moderately Hard	96.0	60.0	60.0	4.0	7.4-7.8	80-100	57-64
Hard	192.0	120.0	120.0	8.0	7.6-8.0	160-180	110-120
Very hard	384.0	240.0	240.0	16.0	8.0-8.4	280-320	225-245

[1]Taken in part from Marking and Dawson (1973).
[2]Add reagent grade chemicals to deionized water.
[3]Approximate equilibrium pH after 24 h of aeration.
[4]Expressed as mg $CaCO_3$/L.

TABLE 8. PREPARATION OF SYNTHETIC FRESHWATER USING MINERAL WATER[1]

Water Type	Volume of Mineral Water Added (mL/L)[2]	Proportion of Mineral Water (%)	Approximate Final Water Quality		
			pH[3]	Hardness[4]	Alkalinity[4]
Very Soft	50	2.5	7.2-8.1	10-13	10-13
Soft	100	10.0	7.9-8.3	40-48	30-35
Moderately Hard	200	20.0	7.9-8.3	80-100	57-64
Hard	400	40.0	7.9-8.3	160-180	110-120
Very Hard[5]	---	---	---	---	---

[1]From Mount et al., 1987; data provided by Philip Lewis, EMSL-Cincinnati.
[2]Add mineral water to MILLI-Q® water or equivalent to prepare DMW (Diluted Mineral Water).
[3]Approximate equilibrium pH after 24 h of aeration.
[4]Expressed as mg $CaCO_3$/L.
[5]Dilutions of PERRIER® Water form a precipitate when concentrations equivalent to "very hard water" are aerated.

TABLE 9. PREPARATION OF SYNTHETIC SEAWATER USING REAGENT GRADE CHEMICALS[1,2,3]

Compound	Concentration (g/L)	Amount (g) Required for 20 L
NaCl	21.03	420.6
Na_2SO_4	3.52	70.4
Kcl	0.61	12.2
Kbr	0.088	1.76
$Na_2B_4O_7 \bullet 10\ H_2O$	0.034	0.68
$MgCl_2 \bullet 6\ H_2O$	9.50	190.0
$CaCl_2 \bullet 2\ H_2O$	1.32	26.4
$SrCl_2 \bullet 6\ H_2O$	0.02	0.400
$NaHCO_3$	0.17	3.40

[1]Modified GP2.
[2]The constituent salts and concentrations were taken from USEPA, 1990b. The salinity is 30.89 G/L.
[3]GP2 can be diluted with deionized (DI) water to the desired test salinity.

7.3.6 When receiving water is used as dilution water in flow-through tests, it is preferable to pump the dilution water continuously to the acclimation chamber and/or dilutor. However, where it is not feasible to pump the dilution water continuously, grab samples of the dilution water are transported to the test site in tanks, and continuously pumped from the tanks to the acclimation chamber and/or dilutor.

7.3.7 HYPERSALINE BRINE

7.3.7.1 Hypersaline brine (HSB) has several advantages that make it desirable for use in toxicity testing. It can be made from any high quality, filtered seawater by evaporation, and can be added to deionized water to prepare dilution water, or to effluents or surface waters to increase their salinity.

7.3.7.2 The ideal container for making HSB from natural seawater is one that (1) has a high surface to volume ratio, (2) is made of a non-corrosive material, and (3) is easily cleaned (fiberglass containers are ideal). Special care should be used to prevent any toxic materials from coming in contact with the seawater being used to generate the brine. If a heater is immersed directly into the seawater, ensure that the heater materials do not corrode or leach any substances that would contaminate the brine. One successful method used is a thermostatically controlled heat exchanger made from fiberglass. If aeration is used, use only oil-free air compressors to prevent contamination.

7.3.7.3 Before adding seawater to the brine generator, thoroughly clean the generator, aeration supply tube, heater, and any other materials that will be in direct contact with the brine. A good quality biodegradable detergent should be used, followed by several thorough deionized water rinses. High quality (and preferably high salinity) seawater should be filtered to at least 10 µm before placing into the brine generator. Water should be collected on an incoming tide to minimize the possibility of contamination.

7.3.7.4 The temperature of the seawater is increased slowly to 40°C. The water should be aerated to prevent temperature stratification and to increase water evaporation. The brine should be checked daily (depending on the volume being generated) to ensure that the salinity does not exceed 100‰ and that the temperature does not exceed 40°C. Additional seawater may be added to the brine to obtain the volume of brine required.

7.3.7.5 After the required salinity is attained, the HSB should be filtered a second time through a 1-μm filter and poured directly into portable containers (20 L CUBITAINERS® or polycarbonate water cooler jugs are suitable). The containers should be capped and labeled with the date the brine was generated and its salinity. Containers of HSB should be stored in the dark and maintained under room temperature until used.

7.3.7.6 If a source of HSB is available, test solutions can be made by following the directions below. Thoroughly mix together the deionized water and brine before mixing in the effluent.

7.3.7.7 Divide the salinity of the HSB by the expected test salinity to determine the proportion of deionized water to brine. For example, if the salinity of the brine is 100‰ and the test is to be conducted at 25‰, 100‰ divided by 25‰ = 4.0. The proportion of brine is 1 part in 4 (one part brine to three parts deionized water).

7.3.7.8 To make 1 L of seawater at 25‰ salinity from a hypersaline brine of 100‰, 250 mL of brine and 750 mL of deionized water are required.

7.4 **USE OF TAP WATER AS DILUTION WATER**

7.4.1 The use of tap water as dilution water is discouraged unless it is dechlorinated and fully treated. Tap water can be dechlorinated by deionization, carbon filtration, or the use of sodium thiosulfate. Use of 3.6 mg/L (anhydrous) sodium thiosulfate will reduce 1.0 mg chlorine/L (APHA, 1992, p. 4-36). Following dechlorination, total residual chlorine should not exceed 0.01 mg/L. Because of the possible toxicity of thiosulfate to test organisms, a control lacking thiosulfate should be included in toxicity tests utilizing thiosulfate-dechlorinated water.

7.4.2 To be adequate for general laboratory use following dechlorination, the tap water is passed through a deionizer and carbon filter to remove toxic metals and organics, and to control hardness and alkalinity.

7.5 **DILUTION WATER HOLDING**

7.5.1 A given batch of dilution water should not be used for more than 14 days following preparation because of the possible build-up of bacterial, fungal, or algal slime growth and the problems associated with it. The container should be kept covered and the contents should be protected from light.

SECTION 8

EFFLUENT AND RECEIVING WATER SAMPLING AND SAMPLE HANDLING

8.1 EFFLUENT SAMPLING

8.1.1 The effluent sampling point is ordinarily the same as that specified in the NPDES discharge permit (USEPA, 1979c). Conditions for exception would be: (1) better access to a sampling point between the final treatment and the discharge outfall; (2) if the effluent is chlorinated prior to discharge to the receiving waters, it may also be desirable to take samples prior to contact with the chlorine to determine toxicity of the unchlorinated effluent; or (3) in the event there is a desire to evaluate the toxicity of the influent to publicly owned treatment works or separate process waters in industrial facilities prior to their being combined with other process waters or non-contact cooling water, additional sampling points may be chosen.

8.1.2 The decision on whether to collect grab or composite samples is based on the requirements of the NPDES permit, the objectives of the test, and an understanding of the short and long-term operations and schedules of the discharger. If the effluent quality varies considerably with time, which can occur where holding times within the treatment facility are short, grab samples may seem preferable because of the ease of collection and the potential of observing peaks (spikes) in toxicity. However, the sampling duration of a grab sample is so short that full characterization of an effluent over a 24-h period would require a prohibitive number of separate samples and tests. Collection of a 24-h composite sample, however, may dilute toxicity spikes, and average the quality of the effluent over the sampling period. Sampling recommendations are provided below.

8.1.3 Aeration during collection and transfer of effluents should be minimized to reduce the loss of volatile chemicals.

8.1.4 Details of date, time, location, duration, and procedures used for effluent sample and dilution water collection should be recorded.

8.2 EFFLUENT SAMPLE TYPES

8.2.1 The advantages and disadvantages of effluent grab and composite samples are listed below:

8.2.1.1 Grab Samples

Advantages:

1. Easy to collect; require a minimum of equipment and on-site time.
2. Provide a measure of instantaneous toxicity. Toxicity spikes are not masked by dilution.

Disadvantages:

1. Samples are collected over a very short period of time and on a relatively infrequent basis. The chances of detecting a spike in toxicity would depend on the frequency of sampling, and the probability of missing spikes is high.

8.2.1.2 Composite Samples:

Advantages:

1. A single effluent sample is collected over a 24-h period.
2. The sample is collected over a much longer period of time than grab samples and contains all toxicity spikes.

Disadvantages:

1. Sampling equipment is more sophisticated and expensive, and must be placed on-site for at least 24 h.
2. Toxicity spikes may not be detected because they are masked by dilution with less toxic wastes.

8.3 EFFLUENT SAMPLING RECOMMENDATIONS

8.3.1 When tests are conducted on-site, test solutions can be renewed daily with freshly collected samples.

8.3.2 When tests are conducted off-site, samples are collected once, or daily, and used for test initiation and renewal.

8.3.3 Sufficient sample must be collected to perform the required toxicity and chemical tests. A 4-L (1-gal) CUBITAINER® will provide sufficient sample volume for most tests (see Tables 12-19).

8.3.4 The following effluent sampling methods are recommended:

8.3.4.1 Continuous Discharges

1. If the facility discharge is continuous, but the calculated retention time of the continuously discharged effluent is less than 14 days and the variability of the effluent toxicity is unknown, at a minimum, four grab samples or four composite samples are collected over a 24-h period. For example, a grab sample is taken every 6 h (total of four samples) and each sample is used for a separate toxicity test, or four successive 6-h composite samples are taken and each is used in a separate test.

2. If the calculated retention time of a continuously discharged effluent is greater than 14 days, or if it can be demonstrated that the wastewater does not vary more than 10% in toxicity over a 24-h period, regardless of retention time, a single grab sample is collected for a single toxicity test.

3. The retention time of the effluent in the wastewater treatment facility may be estimated from calculations based on the volume of the retention basin and rate of wastewater inflow. However, the calculated retention time may be much greater than the actual time because of short-circuiting in the holding basin. Where short-circuiting is suspected, or sedimentation may have reduced holding basin capacity, a more accurate estimate of the retention time can be obtained by carrying out a dye study.

8.3.4.2 Intermittent Discharges

8.3.4.2.1 If the facility discharge is intermittent, a grab sample is collected midway during each discharge period. Examples of intermittent discharges are:

1. When the effluent is continuously discharged during a single 8-h work shift (one sample is collected), or two successive 8-h work shifts (two samples are collected).

2. When the facility retains the wastewater during an 8-h work shift, and then treats and releases the wastewater as a batch discharge (one sample is collected).

3. When the facility discharges wastewater to an estuary only during an outgoing tide, usually during the 4 h following slack high tide (one sample is collected).

8.3.4.3 At the end of a shift, clean up activities may result in the discharge of a slug of toxic waste, which may require sampling and testing.

8.4 RECEIVING WATER SAMPLING

8.4.1 Logistical problems and difficulty in securing sampling equipment generally preclude the collection of composite receiving water samples for toxicity tests. Therefore, it is common practice to collect a single grab sample and use it throughout the test.

8.4.2 The sampling point is determined by the objectives of the test. In rivers, grab samples should be collected at mid-stream and mid-depth, if accessible. At estuarine and marine sites, samples should be collected at mid-depth.

8.4.3 To determine the extent of the zone of toxicity in the receiving water downstream from the outfall, receiving water samples are collected at several distances downstream from the discharge. The time required for the effluent-receiving-water mixture to travel to sampling points downstream from the outfall, and the rate and degree of mixing, may be difficult to ascertain. Therefore, it may not be possible to correlate downstream toxicity with effluent toxicity at the discharge point unless a dye study is performed. The toxicity of receiving water samples from five stations downstream from the discharge point can be evaluated using the same number of test vessels and test organisms as used in one effluent toxicity test with five effluent dilutions.

8.5 EFFLUENT AND RECEIVING WATER SAMPLE HANDLING, PRESERVATION, AND SHIPPING

8.5.1 Unless the samples are used in an on-site toxicity test the day of collection (or hand delivered to the testing laboratory for use on the day of collection), it is recommended that they be held at 0-6°C until used to inhibit microbial degradation, chemical transformations, and loss of highly volatile toxic substances.

8.5.2 Composite samples should be chilled as they are collected. Grab samples should be chilled immediately following collection.

8.5.3 If the effluent has been chlorinated, total residual chlorine must be measured immediately following sample collection.

8.5.4 Sample holding time begins when the last grab sample in a series is taken (i.e., when a series of four grab samples are taken over a 24-h period), or when a 24-h composite sampling period is completed. If the data from the samples are to be acceptable for use in the NPDES Program, the lapsed time (holding time) from sample collection to first use of each grab or composite sample must not exceed 36 h. EPA believes that 36 h is adequate time to deliver the samples to the laboratories performing the tests in most cases. In the isolated cases, where the permittee can document that this delivery time cannot be met, the permitting authority can allow an option for on-site testing or a variance for an extension of shipped sample holding time. The request for a variance in sample holding time, directed to the USEPA Regional Administrator under 40 CFR 136.3(e) should include supportive data which show that the toxicity of the effluent sample is not reduced (e.g., because of volatilization and/or sorption of toxics on the sample container surfaces) by extending the holding time beyond more than 36 h. However, in no case should more than 72 h elapse between collection and first use of the sample. In static-renewal tests, each grab or composite sample may also be used to prepare test solutions for renewal at 24 h, 48 h, and/or 72 h after first use, if stored at 0-6°C, with minimum head space, as described in Subsection 8.5. Guidance for determining the persistence of the sample is provided in Subsection 8.7.

8.5.5 To minimize the loss of toxicity due to volatilization of toxic constituents, all sample containers should be "completely" filled, leaving no air space between the contents and the lid.

8.5.6 SAMPLES USED IN ON-SITE TESTS

8.5.6.1 Samples collected for on-site tests should be used within 24 h.

8.5.7 SAMPLES SHIPPED TO OFF-SITE FACILITIES

8.5.7.1 Samples collected for off-site toxicity testing are to be chilled to 0-6°C during or immediately after collection, and shipped iced to the performing laboratory. Sufficient ice should be placed with the sample in the shipping container to ensure that ice will still be present when the sample arrives at the laboratory and is unpacked. Insulating material should not be placed between the ice and the sample in the shipping container unless required to prevent breakage of glass sample containers.

8.5.7.2 Samples may be shipped in one or more 4-L (1 gal) CUBITAINERS® or new plastic "milk" jugs. All sample containers should be rinsed with source water before being filled with sample. After use with receiving water or effluents, CUBITAINERS® and plastic jugs are punctured to prevent reuse.

8.5.7.3 Several sample shipping options are available, including Express Mail, air express, bus, and courier service. Express Mail is delivered seven days a week. Saturday and Sunday shipping and receiving schedules of private carriers vary with the carrier.

8.6 **SAMPLE RECEIVING**

8.6.1 Upon arrival at the laboratory, samples are logged in and the temperature is measured and recorded. If the samples are not immediately prepared for testing, they are stored at 0-6°C until used.

8.6.2 Every effort must be made to initiate the test with an effluent sample on the day of arrival in the laboratory, and the sample holding time should not exceed 36 h before first use unless a variance has been granted by the NPDES permitting authority.

8.7 **PERSISTENCE OF EFFLUENT TOXICITY DURING SAMPLE SHIPMENT AND HOLDING**

8.7.1 The persistence of the toxicity of an effluent prior to its use in a toxicity test is of interest in assessing the validity of toxicity test data, and in determining the possible effects of allowing an extension of the holding time. Where a variance in holding time (>36 h, but #72 h) is requested by a permittee (see Subsection 8.5.4 above), information on the effects of the extension in holding time on the toxicity of the samples must be obtained by comparing the results of multi-concentration acute toxicity tests performed on effluent samples held 36 h with toxicity test results using the same samples after they were held for the requested, longer period. The portion of the sample set aside for the second test must be held under the same conditions as during shipment and holding.

SECTION 9

ACUTE TOXICITY TEST PROCEDURES

9.1 PREPARATION OF EFFLUENT AND RECEIVING WATER SAMPLES FOR TOXICITY TESTS

9.1.1 When aliquots are removed from the sample container, the head space above the remaining sample should be held to a minimum. Air which enters a container upon removal of sample should be expelled by compressing the container before reclosing, if possible (i.e., where a CUBITAINER® used), or by using an appropriate discharge valve (spigot).

9.1.2 It may be necessary to first coarse-filter samples through a sieve having 2-4 mm mesh openings to remove debris and/or break up large floating or suspended solids. If samples contain indigenous organisms that may attack or be confused with the test organisms, the samples must be filtered through a sieve with 60 µm mesh openings. Caution: filtration may remove some toxicity.

9.1.3 At a minimum, pH, conductivity or salinity, and total residual chlorine are measured in the undiluted effluent or receiving water, and pH and conductivity are measured in the dilution water.

9.1.4 It is recommended that total alkalinity and total hardness also be measured in the undiluted test water (effluent or receiving water) and the dilution water.

9.1.5 Total ammonia is measured in effluent and receiving water samples where toxicity may be contributed by unionized ammonia (i.e., where total ammonia ≥5 mg/L). The concentration (mg/L) of unionized (free) ammonia in a sample is a function of temperature and pH, and is calculated using the percentage value obtained from Table 10, under the appropriate pH and temperature, and multiplying it by the concentration (mg/L) of total ammonia in the sample.

9.1.6 Effluents and receiving waters can be dechlorinated using 6.7 mg/L anhydrous sodium thiosulfate to reduce 1 mg/L chlorine (Standard Methods, 18th Edition, APHA, 1992, p. 9-32; note that the amount of thiosulfate required to dechlorinate effluents is greater than the amount needed to dechlorinate tap water). Since thiosulfate may contribute to sample toxicity, a thiosulfate control should be used in the test in addition to the normal dilution water control.

9.1.7 The DO concentration in the samples should be near saturation prior to use. Aeration may be used to bring the DO and other gases into equilibrium with air, minimize oxygen demand, and stabilize the pH. However, aeration during collection, transfer, and preparation of samples should be minimized to reduce the loss of volatile chemicals.

9.1.8 If the samples must be warmed to bring them to the prescribed test temperature, supersaturation of the dissolved oxygen and nitrogen may become a problem. To avoid this problem, samples may be warmed slowly in open test containers. If DO is still above 100% saturation after warming to test temperature, samples should be aerated moderately (approximately 500 mL/minimum) for a few minutes using an airstone. If DO is below 4.0 mg/L after warming to test temperature, the solutions must be aerated moderately (approximately 500 mL/min) for a few minutes, using an airstone, until the DO is within the prescribed range (≥4.0 mg/L when using warm water species, or ≥6.0 mg/L when using cold water species). Caution: avoid excessive aeration.

9.1.9 Mortality due to pH alone may occur if the pH of the sample falls outside the range of 6.0-9.0. Thus, the presence of other forms of toxicity (metals and organics) in the sample may be masked by the toxic effects of low or high pH. The question about the presence of other toxicants can be answered only by performing two parallel tests, one with an adjusted pH, and one without an adjusted pH. Freshwater samples are adjusted to pH 7.0, and marine samples are adjusted to pH 8.0, by adding 1N NaOH or 1N HCl dropwise, as required, being careful to avoid overadjustment.

TABLE 10. PERCENT UNIONIZED NH_3 IN AQUEOUS AMMONIA SOLUTIONS: TEMPERATURES 15-26EC AND pH's 6.0-8.9[1]

pH	Temperature (°C)											
	15	16	17	18	19	20	21	22	23	24	25	26
6.0	0.0274	0.0295	0.0318	0.0343	0.0369	0.0397	0.0427	0.0459	0.0493	0.0530	0.0568	0.0610
6.1	0.0345	0.0372	0.0400	0.0431	0.0464	0.0500	0.0537	0.0578	0.0621	0.0667	0.0716	0.0768
6.2	0.0434	0.0468	0.0504	0.0543	0.0584	0.0629	0.0676	0.0727	0.0781	0.0901	0.0901	0.0966
6.3	0.0546	0.0589	0.0634	0.0683	0.0736	0.0792	0.0851	0.0915	0.0983	0.1134	0.1134	0.1216
6.4	0.0687	0.0741	0.0799	0.0860	0.0926	0.0996	0.107	0.115	0.124	0.133	0.143	0.153
6.5	0.0865	0.0933	0.1005	0.1083	0.1166	0.1254	0.135	0.145	0.156	0.167	0.180	0.193
6.6	0.109	0.117	0.127	0.136	0.147	0.158	0.170	0.182	0.196	0.210	0.226	0.242
6.7	0.137	0.148	0.159	0.171	0.185	0.199	0.214	0.230	0.247	0.265	0.284	0.305
6.8	0.172	0.186	0.200	0.216	0.232	0.250	0.269	0.289	0.310	0.333	0.358	0.384
6.9	0.217	0.234	0.252	0.271	0.292	0.314	0.338	0.363	0.390	0.419	0.450	0.482
7.0	0.273	0.294	0.317	0.342	0.368	0.396	0.425	0.457	0.491	0.527	0.566	0.607
7.1	0.343	0.370	0.399	0.430	0.462	0.497	0.535	0.575	0.617	0.663	0.711	0.762
7.2	0.432	0.466	0.502	0.540	0.581	0.625	0.672	0.722	0.776	0.833	0.893	0.958
7.3	0.543	0.586	0.631	0.679	0.731	0.786	0.845	0.908	0.975	1.05	1.12	1.20
7.4	0.683	0.736	0.793	0.854	0.918	0.988	1.061	1.140	1.224	1.31	1.41	1.51
7.5	0.858	0.925	0.996	1.07	1.15	1.24	1.33	1.43	1.54	1.65	1.77	1.89
7.6	1.08	1.16	1.25	1.35	1.45	1.56	1.67	1.80	1.93	2.07	2.21	2.37
7.7	1.35	1.46	1.57	1.69	1.82	1.95	2.10	2.25	2.41	2.59	2.77	2.97
7.8	1.70	1.83	1.97	2.12	2.28	2.44	2.62	2.82	3.02	3.24	3.46	3.71
7.9	2.13	2.29	2.46	2.65	2.85	3.06	3.28	3.52	3.77	4.04	4.32	4.62
8.0	2.66	2.87	3.08	3.31	3.56	3.82	4.10	4.39	4.70	5.03	5.38	5.75
8.1	3.33	3.58	3.85	4.14	4.44	4.76	5.10	5.46	5.85	6.25	6.68	7.14
8.2	4.16	4.47	4.80	5.15	5.52	5.92	6.34	6.78	7.25	7.75	8.27	8.82
8.3	5.18	5.56	5.97	6.40	6.86	7.34	7.85	8.39	8.96	9.56	10.2	10.9
8.4	6.43	6.90	7.40	7.93	8.48	9.07	9.69	10.3	11.0	11.7	12.5	13.3
8.5	7.97	8.54	9.14	9.78	10.45	11.16	11.90	12.7	13.5	14.4	15.2	16.2
8.6	9.83	10.5	11.2	12.0	12.8	13.6	14.5	15.5	16.4	17.4	18.5	19.5
8.7	12.07	12.9	13.8	14.7	15.6	16.6	17.6	18.7	19.8	21.0	22.2	23.4
8.8	14.7	15.7	16.7	17.8	18.9	20.0	21.2	22.5	23.7	25.1	26.4	27.8
8.9	17.9	19.0	20.2	21.4	22.7	24.0	25.3	26.7	28.2	29.6	31.1	32.6

[1]Table provided by Teresa Norberg-King, Environmental Research Laboratory, Duluth, Minnesota. Also see Emerson, et. al., 1975, Thurston, et. al, 1974, and USEPA, 1985.

9.2 PRELIMINARY TOXICITY RANGE-FINDING TESTS

9.2.1 USEPA Regional and State personnel generally have observed that it is not necessary to conduct a toxicity range-finding test prior to initiating a static, acute, definitive toxicity test. However, when preparing to perform a static test with an sample of completely unknown quality, or before initiating a flow-through test, it is advisable to conduct a preliminary toxicity range-finding test.

9.2.2 A toxicity range-finding test ordinarily consists of a down-scaled, abbreviated static acute test in which groups of five organisms are exposed to several widely-spaced sample dilutions in a logarithmic series, such as 100%, 10.0%, 1.00%, and 0.100%, and a control, for 8-24 h. **Caution**: if the sample must also be used for the full-scale definitive test, the 36-h limit on holding time (Section 8, , Effluent and Receiving Water Sampling and Sample Handling, Subsection 8.5.4) must not be exceeded before the definitive test is initiated.

9.2.3 It should be noted that the toxicity (LC50) of a sample observed in a range-finding test may be significantly different from the toxicity observed in the follow-up definitive test because: (1) the definitive test is usually longer; and (2) the test may be performed with a sample collected at a different time, and possibly differing significantly in the level of toxicity.

9.3 MULTI-CONCENTRATION (DEFINITIVE) EFFLUENT TOXICITY TESTS

9.3.1 The tests recommended for use in determining discharge permit compliance in the NPDES program are multi-concentration, or definitive, tests which provide (1) a point estimate of effluent toxicity in terms of a LC50, or (2) a no-observed-adverse-effect concentration (NOAEC) defined in terms of mortality, and obtained by hypothesis testing. The tests may be static non-renewal, static renewal, or flow-through.

9.3.2 The tests consist of a control and a minimum of five effluent concentrations. USEPA recommends the use of a $\geq$0.5 dilution factor for selecting effluent test concentrations. Effluent test concentrations of 6.25%, 12.5%, 25%, 50%, and 100% are commonly used, however, test concentrations should be selected independently for each test based on the objective of the study, the expected range of toxicity, the receiving water concentration, and any available historical testing information on the effluent. USEPA (2000a) provides additional guidance on choosing appropriate test concentrations.

9.3.3 When these tests are used in determining compliance with permit limits, effluent test concentrations should be selected to bracket the receiving water concentration (RWC). This may be achieved by selecting effluent test concentrations in the following manner: (1) 100% effluent, (2) [RWC + 100]/2, (3) RWC, (4) RWC/2, and (5) RWC/4. For example, where the RWC = 50%, appropriate effluent concentrations may be 100%, 75%, 50%, 25%, and 12.5%.

9.3.4 If acute/chronic ratios are to be determined by simultaneous acute and short-term chronic tests with a single species, using the same sample, both types of tests must use the same test conditions, i.e., temperature, water hardness, salinity, etc.

9.4 RECEIVING WATER TESTS

9.4.1 Receiving water toxicity tests generally consist of 100% receiving water and a control. The total hardness or salinity of the control should be comparable to the receiving water.

9.4.2 The data from the two treatments are analyzed by hypothesis testing to determine if test organism survival in the receiving water differs significantly from the control. A minimum of four replicates and 10 organisms per replicate are required for each treatment (see Tables 12-19).

9.4.3 In cases where the objective of the test is to estimate the degree of toxicity of the receiving water, a definitive, multi-concentration test is performed by preparing dilutions of the receiving water, using a ≥0.5 dilution series, with a suitable control water.

9.5 **STATIC TESTS**

9.5.1 Static tests may be non-renewal or renewal.

9.5.2 An excess volume of each dilution is prepared to provide sufficient material for toxicity testing and routine chemical analyses. The solutions are well mixed with a glass rod, TEFLON® stir bar, or other means. Aliquots of each sample concentration are delivered to the test chambers, and the chambers are arranged in random order. The test solutions are brought to the required temperature, and the test organisms are added. The remaining volumes of each sample concentration are used, as necessary, for the chemical analyses.

9.5.3 Saline dilution water can be prepared by adding dry salts (FORTY FATHOMS® or equivalent, or modified GP2) or hypersaline brine to de-ionized water, or a suitable surface freshwater, to adjust the salinity of the entire dilution series. If saline receiving water is used as the diluent, a salinity control must be prepared using deionized water and dried sea salts to determine if the addition of sea salts alone has an adverse effect on the test organisms. It may be desirable to conduct static toxicity tests at several salinities.

9.5.4 If the effluent has low salinity, but the test is to be conducted with a salt water organism, the test solutions may be prepared by adding dry ocean salts or hypersaline brine to a sufficient quantity of 100% effluent to raise the salinity to the required level, which will depend on the objectives of the test and the policy of the regulatory agency. After the addition of the dried salts, stir gently for 30 to 60 min, preferably with a magnetic stirrer, to ensure that the salts are in solution. It is important to check the final salinity with a refractometer.

9.5.5 Addition of dry salts to effluents and dilution water may change the pH and affect the toxicity of the waste. If the objective of the test is to determine the toxicity of the effluent at the original pH, the pH of the salinity-adjusted solutions can be brought to the required level by dropwise addition of 1N HCl or 1N NaOH. It is recommended that a concurrent test be conducted with salinity-adjusted effluent in which the pH has not been altered after adding the salt.

9.5.6 The volume of the effluent used must be sufficient to prepare all percent concentrations of the effluent needed for the toxicity test and for routine chemical analysis. For example, to conduct tests with *Menidia*, the use of 200 mL of test solution in each of duplicate exposure vessels and five concentrations of effluent (10 exposure vessels), would require a total of 1 L of 100% effluent. However, to provide sufficient volumes of test solutions for routine chemical analysis and for toxicity testing, additional effluent would be required (1.5-2.0 L).

9.5.7 A standard control lacking thiosulfate should be included in tests where the dilution water was prepared by dechlorinating tap water with thiosulfate.

9.5.8 If, within 1 h of the start of the test, 100% mortality has occurred in the higher effluent concentrations (such as 100% and 50%), additional concentrations of effluents, such as 3.1%, 1.6%, and 0.8%, are added to the test at the lower end of the concentration series.

9.5.9 pH drift during acute, static-renewal, or non-renewal toxicity tests may contribute to artifactual toxicity when ammonia or other pH-dependent toxicants (such as metals) are present. This problem can be minimized by conducting a test in a static-renewal mode rather than a non-renewal mode, or the problem can be avoided by conducting the test in a flow-through mode, rather than a static-renewal or non-renewal mode.

9.6 **FLOW-THROUGH TESTS**

9.6.1 Flow-through tests are usually performed with the same effluent concentrations that are used for static tests, except that where the receiving water is saline and the effluent is not, 100% effluent cannot be tested with a marine organism. Examples of flow-through test systems are provided in the Appendix. Small organisms, such as mysids and daphnids, are confined in screened enclosures placed in the flow-through chambers. More than one species may be used in the same test chamber in a given test, if segregated.

9.6.2 The dilutor system should be operated long enough prior to adding the test organisms to calibrate the dilutor and make the necessary adjustments in the temperature, flow rate through the test chambers, and aeration. The flow rate through the proportional dilutor must provide for a minimum of five 90% replacements of water volume in each test chamber every 24 h (see Figure 2). This replacement rate should provide sufficient flow to maintain an adequate concentration of dissolved oxygen. The dilutor should also be capable of maintaining the test concentration at each dilution within 5% of the starting concentration for the duration of the test. The calibration of the dilutor should be checked carefully before the test begins to determine the volume of effluent and dilution water used in each portion of the effluent delivery system and the flow rate through each test chamber. The general operation of the dilutor should be checked at least at the beginning and end of each day during the test.

9.6.3 The control consists of the same dilution water, test conditions, procedures, and organisms used in testing the effluent. In the event a test is to be conducted with salt water organisms, where each effluent dilution has a different salinity, a static control is prepared for the lowest (or highest, in the case of high salinity, e.g. brine wastes) salinity level used in the flow-through test to determine if salinity alone has any adverse effects on the test organisms.

9.7 **NUMBER OF TEST ORGANISMS**

9.7.1 A minimum of 20 organisms of a given species are exposed to each effluent concentration (Jensen, 1972). Small fish and invertebrates are captured with 4- to 8-mm inside diameter pipettes. Organisms larger than 10 mm can be captured by dip net. In a typical toxicity test involving five effluent concentrations and a control (six concentrations x 20 organisms per concentration), fish and other large test organisms are captured from a common pool and distributed sequentially to the test chambers until the required number of organisms are placed in each. The test chambers are then positioned randomly. To avoid carryover of excess culture water in transferring small organisms to the test chambers, it may be advantageous to distribute small organisms, such as daphnids, mysids, and larval fish, first to small holding vessels, such as weighing boats, petri dishes, or small beakers. The water in the intermediary holding vessels is then drawn down to a small volume and the entire lot is transferred to a test chamber. In the case of daphnids, both excessive handling and carryover of culture water and can be avoided by placing the tip of the transfer pipettes below the surface of the water in the test chambers and allowing the organisms to swim out of the pipettes without discharging the contents.

9.8 **REPLICATE TEST CHAMBERS**

9.8.1 Two or more test chambers are provided for each effluent concentration and the control. Although the data from duplicate chambers are usually combined to determine the LC50 and confidence interval, the practice of dividing the test population for each effluent concentration between two or more replicate chambers has several advantages and is considered good laboratory practice because it: (1) permits easier viewing and counting of test organisms; (2) more easily avoids possible violations of loading limits, which might occur if all of the test organisms are placed in a single test vessel; and (3) ensures against the invalidation of the test which might result from accidental loss of a test vessel, where all of the test organisms for a given treatment are in a single chamber.

9.9 **LOADING OF TEST ORGANISMS**

9.9.1 A limit is placed on the loading (weight) of organisms per liter of test solution to minimize the depletion of dissolved oxygen, the accumulation of injurious concentrations of metabolic waste products, and/or stress induced by crowding, any of which could significantly affect the test results. However, the probability of exceeding loading limits is greatly reduced with the use of very young test organisms.

9.9.2 For both renewal and non-renewal static tests, loading in the test solutions must not exceed the following live weights: 1.1 g/L at 15°C, 0.65 g/L at 20°C, or 0.40 g/L at 25°C.

9.9.3 For flow-through tests, the live weight of test organisms in the test chambers must not exceed 7.0 g/L of test solution at l5°C, or 2.5 g/L at 25°C.

9.10 **ILLUMINATION**

9.10.1 Light of the quality and intensity normally obtained in the laboratory during working hours is adequate (10-20 $\mu E/m^2/s$ or 50-100 ft-c). A uniform photoperiod of 16 h light and 8 h darkness can be achieved in the laboratory or environmental chamber, using automatic timers.

9.11 **FEEDING**

9.11.1 Where indicated in the test summary tables (Tables 12-19), food is made available to test organisms while holding before they are placed in the test chambers. The organisms are fed at test renewal, 48 h after the test is initiated, if Regional or State policy requires a 96-h test duration.

9.11.2 Where *Artemia* nauplii are fed, the nauplii are first concentrated on a NITEX® screen and then are resuspended in fresh or salt water, depending on the salinity of the test solutions, using just enough water to form a slurry that can be transferred by pipette. It should be noted that *Artemia* nauplii placed in freshwater usually die in 4 h, generally are not eaten after death, and decay rapidly, whereas those placed in saline water remain viable and can serve as food for the duration of the test.

9.11.3 Problems caused by feeding, such as the possible alteration of the toxicant concentration, the build-up of food and metabolic wastes and resulting oxygen demand, are common in static test systems. Where feeding is necessary, excess food should be removed daily by aspirating with a pipette.

9.11.4 Feeding does not cause the above problems in flow-through systems. However, it is advisable to remove excess food, fecal material, and any particulate matter that settles from the effluent, from the bottom of the test vessels daily by aspirating with a pipette.

9.12 **TEST TEMPERATURE**

9.12.1 Test temperature will depend on the test species and objectives of the test (see Tables 12-19). Where acute and short-term chronic toxicity tests are performed simultaneously with the same species to determine acute:chronic ratios, both tests must be performed at the chronic test temperature. The average daily temperature of the test solutions should be maintained within ±1°C of the selected test temperature, for the duration of the test. This can be accomplished for static tests by use of a water bath or environmental chamber, and in flow-through tests by passing the effluent and/or dilution water through separate coils immersed in a heating or cooling water bath prior to entering the dilutor system. Coils should be made from materials recommended in Section 5, Facilities and Equipment.

9.13 **STRESS**

9.13.1 Minimize stress on test organisms by avoiding unnecessary disturbances.

9.14 **DISSOLVED OXYGEN CONCENTRATION**

9.14.1 Aeration during the test may alter the results and should be used only as a last resort to maintain the required DO. Aeration can reduce the apparent toxicity of the test solutions by stripping them of highly volatile toxic substances, or increase its toxicity by altering the pH. However, the DO in the test solution should not be permitted to fall below 4.0 mg/L for warm water species and 6.0 mg/L for cold water species. Oxygen saturation values in fresh and saline waters can be determined from Figure 3 and Table 11, respectively.

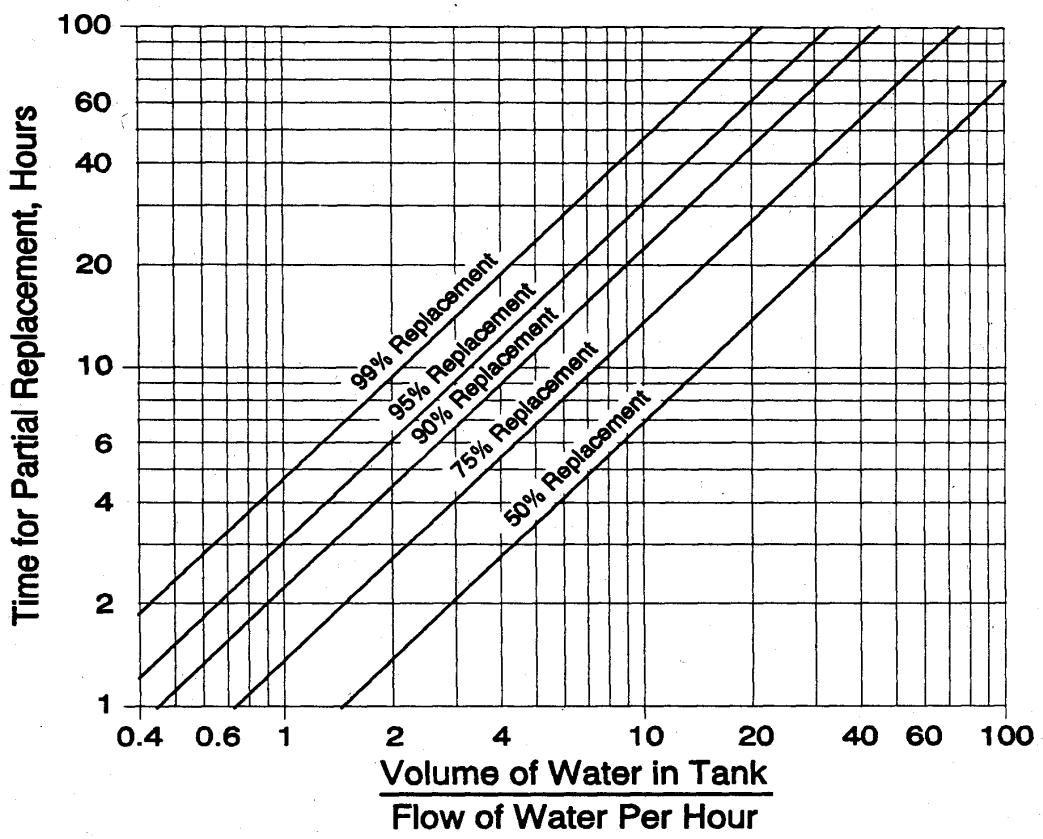

Figure 2. Approximate times required to replace water in test chambers in flow-through tests. For example: for a chamber containing 4 L, with a flow of 2 L/h, the above graph indicates that 90% of the water would be replaced every 4.8 h. The same time period (such as hours) must be used on both axes, and the same unit of volume (such as liters) must be used for both volume and flow (From: Sprague, 1969).

TABLE 11. OXYGEN SOLUBILITY (MG/L) IN WATER AT EQUILIBRIUM WITH AIR AT 760 MM HG (AFTER RICHARDS AND CORWIN, 1956)

Temp	Salinity (‰)									
°C	0	5	10	15	20	25	30	35	40	45
0	14.2	13.8	13.4	12.9	12.5	12.1	11.7	11.2	10.8	10.6
1	13.8	13.4	13.0	12.6	12.2	11.8	11.4	11.0	10.6	10.3
2	13.4	13.0	12.6	12.2	11.9	11.5	11.1	10.7	10.3	10.0
3	13.1	12.7	12.3	11.9	11.6	11.2	10.8	10.4	10.0	9.8
4	12.7	12.3	12.0	11.6	11.3	10.9	10.5	10.1	9.8	9.5
5	12.4	12.0	11.7	11.3	11.0	10.6	10.2	9.8	9.5	9.3
6	12.1	11.7	11.4	11.0	10.7	10.3	10.0	9.6	9.3	9.1
8	11.5	11.2	10.8	10.5	10.2	9.8	9.5	9.2	8.9	8.7
10	10.9	10.7	10.3	10.0	9.7	9.4	9.1	8.8	8.5	8.3
12	10.5	10.2	9.9	9.6	9.3	9.0	8.7	8.4	8.1	7.9
14	10.0	9.7	9.5	9.2	8.9	8.6	8.3	8.1	7.8	7.6
16	9.6	9.3	9.1	8.8	8.5	8.3	8.0	7.7	7.5	7.3
18	9.2	9.0	8.7	8.5	8.2	8.0	7.7	7.5	7.2	7.1
20	8.9	8.6	8.4	8.1	7.9	7.7	7.4	7.2	6.9	6.8
22	8.6	8.4	8.1	7.9	7.6	7.4	7.2	6.9	6.7	6.6
24	8.3	8.1	7.8	7.6	7.4	7.2	6.9	6.7	6.5	6.4
26	8.1	7.8	7.6	7.4	7.2	7.0	6.7	6.5	6.3	6.1
28	7.8	7.6	7.4	7.2	7.0	6.8	6.5	6.3	6.1	6.0
30	7.6	7.4	7.1	6.9	6.7	6.5	6.3	6.1	5.9	5.8
32	7.3	7.1	6.9	6.7	6.5	6.3	6.1	5.9	5.7	5.6

CORRECTION FACTORS FOR OXYGEN SATURATION AT VARIOUS ALTITUDES

ALTITUDE		PRESSURE	
FT	M	MM	FACTOR
0	0	760	1.00
330	100	750	1.01
665	200	741	1.03
980	300	732	1.04
1310	400	723	1.05
1640	500	714	1.06
1970	600	705	1.08
2300	700	696	1.09
2630	800	687	1.11
2950	900	679	1.12
3280	1000	671	1.13
3610	1100	663	1.15
3940	1200	655	1.16
4270	1300	647	1.17
4600	1400	639	1.19
4930	1500	631	1.20
5250	1600	623	1.22
5580	1700	615	1.24
5910	1800	608	1.25
6240	1900	601	1.26
6560	2000	594	1.28
6900	2100	587	1.30
7220	2200	580	1.31
7550	2300	573	1.33
7880	2400	566	1.34
8200	2500	560	1.36

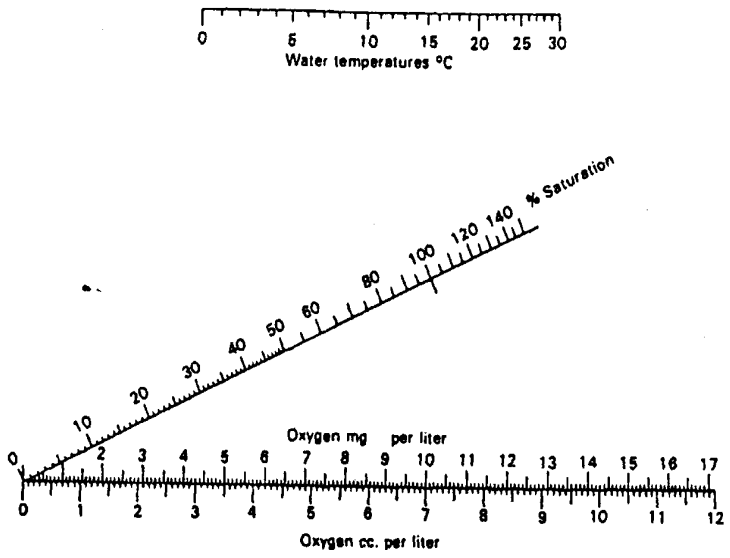

Figure 3. Rawson's nomograph for obtaining oxygen saturation values in freshwater at different temperatures at sea level. When a straightedge is used to connect the water temperature on the upper scale and the concentration on the lower scale, the percent saturation can be read from the point of intersection on the diagonal scale. To determine the percent saturation at locations above sea level, factors are provided to convert oxygen concentrations measured at various altitudes to sea level values in the table at the upper left. For example, an oxygen concentration of 6.4 mg/L measured in a body of water at an altitude of 1000 m and a temperature of 15°C would be equivalent to a concentration of 6.4 x 1.13, or 7.2 mg/L, at sea level. To determine the percent saturation, a straightedge is used to connect the point at 15°C on the temperature scale with the point, 7.2 mg/L on the concentration scale, and the percent saturation is read at the point of intersection (68%) on the diagonal scale. (From Welch, 1948).

9.14.2 In static tests, low DOs commonly occur in the higher concentrations of wastewater. Aeration is accomplished by bubbling air through a pipet at the rate of 100 bubbles/min. If aeration is necessary, all test solutions must be aerated. It is advisable to monitor the DO closely during the first few hours of the test. Samples with a potential DO problem generally show a downward trend in DO within 4 to 8 h after the test is started. Unless aeration is initiated during the first 8 h of the test, the DO may be exhausted during an unattended period, thereby invalidating the test.

9.14.3 In most flow-through tests, DO depletion is not a problem in the test chambers because aeration occurs as the liquids pass through the dilutor system. If the DO decreases to a level that would be a source of additional stress, the turnover rate of the solutions in the test chambers must be increased sufficiently to maintain acceptable DO levels. If the increased turnover rate does not maintain adequate DO levels, aerate the dilution water prior to the addition of the effluent, and aerate all test solutions. To reduce the potential for driving off volatile compounds in the wastewater, aeration may be accomplished by bubbling air through a 1 mL pipet at a rate of no more than 100 bubbles/min, using an air valve to control the flow.

9.14.4 Caution must be exercised to avoid excessive aeration. Turbulence caused by aeration should not result in a physical stress to the test organisms. When aeration is used, the methodology must be detailed in the report. For safety reasons, pure oxygen should not be used to aerate test solutions.

9.15 **TEST DURATION**

9.15.1 Test duration may vary from 24 to 96 h depending on the objectives of the test and the requirements of the regulatory authority. For specific information on test duration, see the tables summarizing the test conditions below.

9.16 **ACCEPTABILITY OF TEST RESULTS**

9.16.1 For the test results to be acceptable, survival in controls must be at least 90%. Tests in which the control survival is less than 90% are invalid, and must be repeated. In tests with specific chemicals, the concentration of the test material must not vary more than 20% at any treatment level during the exposure period.

9.16.2 Upon subsequent completion of a valid test, the results of all tests, valid and invalid, are reported to the regulatory authority with an explanation of the tests performed and results.

9.17 **SUMMARY OF TEST CONDITIONS FOR THE PRINCIPAL TEST ORGANISMS**

9.17.1 Summaries of the test conditions for the daphnids, *Ceriodaphnia dubia*, *Daphnia pulex*, and *D. magna*, fathead minnows, *Pimephales promelas*, rainbow trout, *Oncorhynchus mykiss*, brook trout, *Salvelinus fontinalis*, the mysids, *Mysidopsis bahia* and *Holmesimysis costata*, sheepshead minnows, *Cyprinodon variegatus*, and silversides, *Menidia beryllina, M. menidia*, and *M. peninsulae*, are provided in Tables 12-19.

TABLE 12. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR *CERIODAPHNIA DUBIA* ACUTE TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 2002.0)[1]

1. Test type:	Static non-renewal, static-renewal, or flow-through (available options)
2. Test duration:	24, 48, or 96 h (available options)
3. Temperature:[2]	20°C ±1°C; or 25°C ±1°C (recommended) Test temperatures must not deviate (i.e., maximum minus minimum temperature) by more than 3°C during the test (required)
4. Light quality:	Ambient laboratory illumination (recommended)
5. Light intensity:	10-20 μE/m^2/s (50-100 ft-c) (recommended) (ambient laboratory levels)
6. Photoperiod:	16 h light, 8 h darkness (recommended)
7. Test chamber size:	30 mL (recommended minimum)
8. Test solution volume:	15 mL (recommended minimum)
9. Renewal of test solutions:	After 48 h (required minimum)
10. Age of test organisms:	Less than 24-h old (required)
11. No. organisms per test chamber:	5 for effluent and receiving water tests (required minimum)
12. No. replicate chambers per concentration:	4 for effluent and receiving water tests (required minimum)
13. No. organisms per concentration:	20 for effluent and receiving water tests (required minimum)
14. Feeding regime:	Feed YCT and *Selenastrum* while holding prior to the test; newly-released young should have food available a minimum of 2 h prior to use in a test; add 0.1 mL each of YCT and *Selenastrum* 2 h prior to test solution renewal at 48 h (recommended)
15. Test chamber cleaning:	Cleaning not required
16. Test chamber aeration:	None (recommended)

[1] For the purposes of reviewing WET test data submitted under NPDES permits, each test condition listed above is identified as required or recommended (see Subsection 12.2 for more information on test review). Additional requirements may be provided in individual permits, such as specifying a given test condition where several options are given in the method.

[2] Acute and chronic toxicity tests performed simultaneously to obtain acute/chronic ratios must use the same temperature and dilution water.

TABLE 12. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR *CERIODAPHNIA DUBIA* ACUTE TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 2002.0) (CONTINUED)

17. Dilution water:	Moderately hard synthetic water prepared using MILLIPORE MILLI-Q® or equivalent deionized water and reagent grade chemicals or 20% DMW (see Section 7, Dilution Water), receiving water, ground water, or synthetic water, modified to reflect receiving water hardness (available options)
18. Test concentrations:	Effluents: 5 and a control (required minimum) Receiving Waters: 100% receiving water and a control (recommended)
19. Dilution series:	Effluents: ≥0.5 dilution series (recommended) Receiving Waters: None, or ≥0.5 dilution series (recommended)
20. Endpoint:	Effluents: Mortality (required) Receiving Waters: Mortality (required)
21. Sampling and sample holding requirements:	Effluents: Grab or composite sample first used within 36 h of completion of the sampling period (required) Receiving Waters: Grab or composite sample first used within 36 h of completion of the sampling period (recommended)
22. Sample volume required:	1 L (recommended)
23. Test acceptability criterion:	90% or greater survival in controls (required)

TABLE 13. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR *DAPHNIA PULEX* AND *D. MAGNA* ACUTE TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 2021.0)[1]

1. Test type:	Static non-renewal, static-renewal, or flow-through (available options)
2. Test duration:	24, 48, or 96 h (available options)
3. Temperature:[2]	20°C ±1°C; or 25°C ±1°C (recommended) Test temperatures must not deviate (i.e., maximum minus minimum temperature) by more than 3°C during the test (required)
4. Light quality:	Ambient laboratory illumination (recommended)
5. Light intensity:	10-20 µE/m^2/s (50-100 ft-c) (ambient laboratory levels) (recommended)
6. Photoperiod:	16 h light, 8 h darkness (recommended)
7. Test chamber size:	30 mL (recommended minimum)
8. Test solution volume:	25 mL (recommended minimum)
9. Renewal of test solutions:	After 48 h (required minimum)
10. Age of test organisms:	Less than 24-h old (required)
11. No. organisms per test chamber:	5 for effluent and receiving water tests (required minimum)
12. No. replicate chambers per concentration:	4 for effluent and receiving water tests (required minimum)
13. No. organisms per concentration:	20 for effluent and receiving water tests (required minimum)
14. Feeding regime:	Feed YCT and *Selenastrum* while holding prior to the test; newly-released young should have food available a minimum of 2 h prior to use in a test; add 0.1 mL each of YCT and *Selenastrum* 2 h prior to test solution renewal at 48 h (recommended)
15. Test chamber cleaning:	Cleaning not required
16. Test chamber aeration:	None (recommended)
17. Dilution water:	Moderately hard synthetic water prepared using MILLIPORE MILLI-Q® or equivalent deionized water and reagent grade chemicals or 20% DMW (see Section 7, Dilution Water), receiving water, ground water, or synthetic water, modified to reflect receiving water hardness (available options)

[1] For the purposes of reviewing WET test data submitted under NPDES permits, each test condition listed above is identified as required or recommended (see Subsection 12.2 for more information on test review). Additional requirements may be provided in individual permits, such as specifying a given test condition where several options are given in the method.

[2] Acute and chronic toxicity tests performed simultaneously to obtain acute/chronic ratios must use the same temperature and dilution water.

TABLE 13. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR *DAPHNIA PULEX* AND *D. MAGNA* ACUTE TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATER (TEST METHOD 2021.0) (CONTINUED)

18. Test concentrations:	Effluents: 5 and a control (required minimum) Receiving Waters: 100% receiving water and a control (recommended)
19. Dilution series:	Effluents: ≥0.5 dilution series (recommended) Receiving Waters: None, or ≥0.5 dilution series (recommended)
20. Endpoint:	Effluents: Mortality (required) Receiving Waters: Mortality (required)
21. Sampling and sample holding requirements:	Effluents: Grab or composite sample first used within 36 h of completion of the sampling period (required) Receiving Waters: Grab or composite sample first used within 36 h of completion of the sampling period (recommended)
22. Sample volume required:	1 L (recommended)
23. Test acceptability criterion:	90% or greater survival in controls (required)

TABLE 14. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR FATHEAD MINNOW, *PIMEPHALES PROMELAS*, ACUTE TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 2000.0)[1,2]

1. Test type:	Static non-renewal, static-renewal, or flow-through (available options)
2. Test duration:	24, 48, or 96 h (available options)
3. Temperature:[3]	20°C ±1°C; or 25°C ±1°C (recommended) Test temperatures must not deviate (i.e., maximum minus minimum temperature) by more than 3°C during the test (required)
4. Light quality:	Ambient laboratory illumination (recommended)
5. Light intensity:	10-20 μE/m^2/s (50-100 ft-c) (ambient laboratory levels) (recommended)
6. Photoperiod:	16 h light, 8 h darkness (recommended)
7. Test chamber size:	250 mL (recommended minimum)
8. Test solution volume:	200 mL (recommended minimum)
9. Renewal of test solutions:	After 48 h (required minimum)
10. Age of test organisms:	1-14 days; less than or equal to 24-h range in age (required)
11. No. organisms per test chamber:	10 for effluent and receiving water tests (required minimum)
12. No. replicate chambers per concentration:	2 for effluent tests (required minimum) 4 for receiving water tests (required minimum)
13. No. organisms per concentration:	20 for effluent tests (required minimum) 40 for receiving water tests (required minimum)
14. Feeding regime:	*Artemia* nauplii are made available while holding prior to the test; add 0.2 mL *Artemia* nauplii concentrate 2 h prior to test solution renewal at 48 h (recommended)
15. Test chamber cleaning:	Cleaning not required
16. Test solution aeration:	None, unless DO concentration falls below 4.0 mg/L; rate should not exceed 100 bubbles/min (recommended)

[1] *Cyprinella leedsi* (Bannerfish shiner, formerly *Notropis leedsi*; AFS, 1991) can be used with the test conditions in this table, where it is the required test organism in NPDES permits.

[2] For the purposes of reviewing WET test data submitted under NPDES permits, each test condition listed above is identified as required or recommended (see Subsection 12.2 for more information on test review). Additional requirements may be provided in individual permits, such as specifying a given test condition where several options are given in the method.

[3] Acute and chronic toxicity tests performed simultaneously to obtain acute/chronic ratios must use the same temperature and dilution water.

TABLE 14. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR FATHEAD MINNOW, *PIMEPHALES PROMELAS*, ACUTE TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS[1] (TEST METHOD 2000.0) (CONTINUED)

17. Dilution water:	Moderately hard synthetic water prepared using MILLIPORE MILLI-Q® or equivalent deionized water and reagent grade chemicals or 20% DMW (see Section 7, Dilution Water), receiving water, ground water, or synthetic water, modified to reflect receiving water hardness. (available options)
18. Test concentrations:	Effluents: 5 and a control (required minimum) Receiving Waters: 100% receiving water and a control (recommended)
19. Dilution series:	Effluents: ≥0.5 dilution series (recommended) Receiving Waters: None, or ≥ 0.5 dilution series (recommended)
20. Endpoint:	Effluents: Mortality (required) Receiving Waters: Mortality (required)
21. Sampling and sample holding requirements:	Effluents: Grab or composite sample first used within 36 h of completion of the sampling period (required) Receiving Waters: Grab or composite sample first used within 36 h of completion of the sampling period (recommended)
22. Sample volume required:	2 L for effluents and receiving waters (recommended)
23. Test acceptability criterion:	90% or greater survival in controls (required)

TABLE 15. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR RAINBOW TROUT, *ONCORHYNCHUS MYKISS*, AND BROOK TROUT, *SALVELINUS FONTINALIS*, ACUTE TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 2019.0)[1]

1. Test type:	Static non-renewal, static-renewal, or flow-through (available options)
2. Test duration:	24, 48, or 96 h (available options)
3. Temperature:	12°C ±1°C (recommended) Test temperatures must not deviate (i.e., maximum minus minimum temperature) by more than 3°C during the test (required)
4. Light quality:	Ambient laboratory illumination (recommended)
5. Light intensity:	10-20 μE/m^2/s (50-100 ft-c) (ambient laboratory levels) (recommended)
6. Photoperiod:	16 h light, 8 h darkness. Light intensity should be raised gradually over a 15 min period at the beginning of the photoperiod, and lowered gradually at the end of the photoperiod, using a dimmer switch or other suitable device. (recommended)
7. Test chamber size:	5 L (recommended minimum) (test chambers should be covered to prevent fish from jumping out)
8. Test solution volume:	4 L (recommended minimum)
9. Renewal of test solutions:	After 48 h (required minimum)
10. Age of test organisms:	Rainbow Trout: 15-30 days (after yolk sac absorption to 30 days) (required) Brook Trout: 30-60 days (required)
11. No. organisms per test chamber:	10 for effluent and receiving water tests (required minimum)
12. No. replicate chambers per concentration:	2 for effluent tests (required minimum) 4 for receiving water tests (required minimum)
13. No. organisms per concentration:	20 for effluent tests (required minimum) 40 for receiving water tests (required minimum)
14. Feeding regime:	Feeding not required
15. Test chamber cleaning:	Cleaning not required
16. Test solution aeration:	None, unless DO concentration falls below 6.0 mg/L; rate should not exceed 100 bubbles/min (recommended)

[1] For the purposes of reviewing WET test data submitted under NPDES permits, each test condition listed above is identified as required or recommended (see Subsection 12.2 for more information on test review). Additional requirements may be provided in individual permits, such as specifying a given test condition where several options are given in the method.

TABLE 15. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR RAINBOW TROUT, *ONCORHYNCHUS MYKISS*, AND BROOK TROUT, *SALVELINUS FONTINALIS*, ACUTE TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 2019.0) (CONTINUED)

17. Dilution water:	Moderately hard synthetic water prepared using MILLIPORE MILLI-Q® or equivalent deionized water and reagent grade chemicals or 20% DMW (see Section 7, Dilution Water), receiving water, ground water, or synthetic water, modified to reflect receiving water hardness (available options)
18. Test concentrations:	Effluents: 5 and a control (required minimum) Receiving Waters: 100% receiving water and a control (recommended)
19. Dilution series:	Effluents: ≥ 0.5 dilution series (recommended) Receiving Waters: None, or ≥ 0.5 dilution series (recommended)
20. Endpoint:	Effluents: Mortality (required) Receiving Waters: Mortality (required)
21. Sampling and sample holding requirements:	Effluents: Grab or composite sample first used within 36 h of completion of the sampling period (required) Receiving Waters: Grab or composite sample first used within 36 h of completion of the sampling period (recommended)
22. Sample volume required:	20 L for effluents (recommended) 40 L for receiving waters (recommended)
23. Test acceptability criterion:	90% or greater survival in controls (required)

TABLE 16. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR MYSID, *MYSIDOPSIS BAHIA*, ACUTE TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 2007.0)[1]

1. Test type:	Static non-renewal, static-renewal, or flow-through (available options)
2. Test duration:	24, 48, or 96 h (available options)
3. Temperature[2]:	20°C ±1°C; or 25°C ±1°C (recommended) Test temperatures must not deviate (i.e., maximum minus minimum temperature) by more than 3°C during the test (required)
4. Light quality:	Ambient laboratory illumination (recommended)
5. Light intensity:	10-20 μE/m^2/s (50-100 ft-c) (ambient laboratory levels) (recommended)
6. Photoperiod:	16 h light, 8 h darkness (recommended)
7. Test chamber size:	250 mL (recommended minimum)
8. Test solution volume:	200 mL (recommended minimum)
9. Renewal of test solutions:	After 48 h (required minimum)
10. Age of test organisms:	1-5 days; less than or equal to 24-h range in age (required)
11. No. organisms per test chamber:	10 for effluent and receiving water tests (required minimum)
12. No. replicate chambers per concentration:	2 for effluent tests (required minimum) 4 for receiving water tests (required minimum)
13. No. organisms per concentration:	20 for effluent tests (required minimum) 40 for receiving water tests (required minimum)
14. Feeding regime:	*Artemia* nauplii are made available while holding prior to the test; feed 0.2 mL of concentrated suspension of *Artemia* nauplii ≤ 24-h old, daily (approximately 100 nauplii per mysid) (recommended)

[1] For the purposes of reviewing WET test data submitted under NPDES permits, each test condition listed above is identified as required or recommended (see Subsection 12.2 for more information on test review). Additional requirements may be provided in individual permits, such as specifying a given test condition where several options are given in the method.

[2] Acute and chronic toxicity tests performed simultaneously to obtain acute/chronic ratios must use the same temperature, salinity, and dilution water.

TABLE 16. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR MYSID, *MYSIDOPSIS BAHIA*, ACUTE TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS[1] (TEST METHOD 2007.0) (CONTINUED)

15. Test chamber cleaning:	Cleaning not required
16. Test solution aeration:	None, unless DO concentration falls below 4.0 mg/L; rate should not exceed 100 bubbles/min (recommended)
17. Dilution water:	5-30‰ ±10%; Uncontaminated source of seawater, deionized water mixed with hypersaline brine or artificial sea salts (HW MARINEMIX®, FORTY FATHOMS®, modified GP2, or equivalent) prepared with MILLI-Q® or equivalent deionized water (see Section 7, Dilution Water); or receiving water (available options)
18. Test concentrations:	Effluents: 5 and a control (required minimum) Receiving Waters: 100% receiving water and a control (recommended)
19. Dilution series:	Effluents: ≥0.5 dilution series (recommended) Receiving Waters: None, or ≥0.5 dilution series (recommended)
20. Endpoint:	Effluents: Mortality (required) Receiving Waters: Mortality (required)
21. Sampling and sample holding requirements:	Effluents: Grab or composite sample first used within 36 h of completion of the sampling period (required) Receiving Waters: Grab or composite sample first used within 36 h of completion of the sampling period (recommended)
22. Sample volume required:	1 L for effluents (recommended) 2 L for receiving waters (recommended)
23. Test acceptability criterion:	90% or greater survival in controls (required)

TABLE 17. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS*, ACUTE TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 2004.0)[1]

1. Test type:	Static non-renewal, static-renewal, or flow-through (available options)
2. Test duration:	24, 48, or 96 h (available options)
3. Temperature:[2]	20°C ±1°C; or 25°C ±1°C (recommended) Test temperatures must not deviate (i.e., maximum minus minimum temperature) by more than 3°C during the test (required)
4. Light quality:	Ambient laboratory illumination (recommended)
5. Light intensity:	10-20 µE/m^2/s (50-100 ft-c) (ambient laboratory levels) (recommended)
6. Photoperiod:	16 h light, 8 h darkness (recommended)
7. Test chamber size:	250 mL (recommended minimum)
8. Test solution volume:	200 mL (recommended minimum)
9. Renewal of test solutions:	After 48 h (required minimum)
10. Age of test organisms:	1-14 days; less than or equal to 24-h range in age (required)
11. No. organisms per test chamber:	10 for effluent and receiving water tests (required minimum)
12. No. replicate chambers per concentration:	2 for effluent tests (required minimum) 4 for receiving water tests (required minimum)
13. No. organisms per concentration:	20 for effluent tests (required minimum) 40 for receiving water tests (required minimum)
14. Feeding regime:	*Artemia* nauplii are made available while holding prior to the test; add 0.2 mL *Artemia* nauplii concentrate 2 h prior to test solution renewal at 48 h (recommended)
15. Test chamber cleaning:	Cleaning not required
16. Test solution aeration:	None, unless DO concentration falls below 4.0 mg/L; rate should not exceed 100 bubbles/min (recommended)

[1] For the purposes of reviewing WET test data submitted under NPDES permits, each test condition listed above is identified as required or recommended (see Subsection 12.2 for more information on test review). Additional requirements may be provided in individual permits, such as specifying a given test condition where several options are given in the method.

[2] Acute and chronic toxicity tests performed simultaneously to obtain acute/chronic ratios must use the same temperature, salinity, and dilution water.

TABLE 17. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS*, ACUTE TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 2004.0) (CONTINUED)

17. Dilution water:	5-32‰ ±10%; Uncontaminated source of seawater, deionized water mixed with hypersaline brine or artificial sea salts (HW MARINEMIX®, FORTY FATHOMS®, modified GP2, or equivalent) prepared with MILLI-Q® or equivalent deionized water (see Section 7, Dilution Water); or receiving water (available options)
18. Test concentrations:	Effluents: 5 and a control (required minimum) Receiving Waters: 100% receiving water and a control (recommended)
19. Dilution series:	Effluents: ≥0.5 dilution series (recommended) Receiving Waters: None, or ≥ 0.5 dilution series (recommended)
20. Endpoint:	Effluents: Mortality (required) Receiving Waters: Mortality (required)
21. Sampling and sample holding requirements:	Effluents: Grab or composite sample first used within 36 h of completion of the sampling period (required) Receiving Waters: Grab or composite sample first used within 36 h of completion of the sampling period (recommended)
22. Sample volume required:	1 L for effluents (recommended) 2 L for receiving waters (recommended)
23. Test acceptability criterion:	90% or greater survival in controls (required)

TABLE 18. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR SILVERSIDE, *MENIDIA BERYLLINA, M. MENIDIA*, AND *M. PENINSULAE*, ACUTE TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 2006.0)[1]

1. Test type:	Static non-renewal, static-renewal, or flow-through (available options)
2. Test duration:	24, 48, or 96 h (available options)
3. Temperature:[2]	20°C ±1°C; or 25°C ±1°C (recommended) Test temperatures must not deviate (i.e., maximum minus minimum temperature) by more than 3°C during the test (required)
4. Light quality:	Ambient laboratory illumination (recommended)
5. Light intensity:	10-20 μE/m^2/s (50-100 ft-c) (ambient laboratory levels) (recommended)
6. Photoperiod:	16 h light, 8 h darkness (recommended)
7. Test chamber size:	250 mL (recommended minimum)
8. Test solution volume:	200 mL (recommended minimum)
9. Renewal of test solutions:	After 48 h (required minimum)
10. Age of test organisms:	9-14 days; less than or equal to 24-h range in age (required)
11. No. organisms per test chamber:	10 for effluent and receiving water tests (required minimum)
12. No. replicate chambers per concentration:	2 for effluent tests (required minimum) 4 for receiving water tests (required minimum)
13. No. organisms per concentration:	20 for effluent tests (required minimum) 40 for receiving water tests (required minimum)
14. Feeding regime:	*Artemia* nauplii are made available while holding prior to the test; add 0.2 mL *Artemia* nauplii concentrate 2 h prior to test solution renewal at 48 h (recommended)
15. Test chamber cleaning:	Cleaning not required
16. Test solution aeration:	None, unless DO concentration falls below 4.0 mg/L; rate should not exceed 100 bubbles/min (recommended)

[1] For the purposes of reviewing WET test data submitted under NPDES permits, each test condition listed above is identified as required or recommended (see Subsection 12.2 for more information on test review). Additional requirements may be provided in individual permits, such as specifying a given test condition where several options are given in the method.

[2] Acute and chronic toxicity tests performed simultaneously to obtain acute/chronic ratios must use the same temperature, salinity, and dilution water.

TABLE 18. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR SILVERSIDE, *MENIDIA BERYLLINA, M. MENIDIA*, AND *M. PENINSULAE*, ACUTE TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 2006.0) (CONTINUED)

17. Dilution water:	5-32‰ ±10%; Uncontaminated source of seawater, deionized water mixed with hypersaline brine or artificial sea salts (HW MARINEMIX®, FORTY FATHOMS®, modified GP2, or equivalent) prepared with MILLI-Q® or equivalent deionized water (see Section 7, Dilution Water); or receiving water (available options) 1-32‰ ±10% for *M. beryllina*; 15-32‰ ±10% for *M. menidia*; and *M. peninsulae*
18. Test concentrations:	Effluents: 5 and a control (required minimum) Receiving Waters: 100% receiving water and a control (recommended)
19. Dilution series:	Effluents: ≥0.5 dilution series (recommended) Receiving Waters: None, or ≥0.5 dilution series (recommended)
20. Endpoint:	Effluents: Mortality (required) Receiving Waters: Mortality (required)
21. Sampling and sample holding requirements:	Effluents: Grab or composite sample first used within 36 h of completion of the sampling period (required) Receiving Waters: Grab or composite sample first used within 36 h of completion of the sampling period (recommended)
22. Sample volume required:	1 L for effluents (recommended) 2 L for receiving waters (recommended)
23. Test acceptability criterion:	90% or greater survival in controls (required)

TABLE 19. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR WEST COAST MYSID, *HOLMESIMYSIS COSTATA*, ACUTE TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS[1]

NOTE: This method is specific to Pacific Coast waters and is not listed at 40 CFR Part 136 for nationwide use. This method has been proposed but not yet approved at 40 CFR Part 136.

1. Test type:	Static non-renewal or static-renewal
2. Test duration:	24, 48, or 96 h
3. Temperature:	15°C ± 1°C for organisms collected South of Pt Conception, CA 13°C ± 1°C for organisms collected North of Pt Conception, CA
4. Light quality:	Ambient laboratory illumination
5. Light intensity:	10-20 μE/m^2/s (50-100 ft-c) (ambient laboratory levels)
6. Photoperiod:	16 h light, 8 h darkness
7. Test chamber size:	1000 mL (minimum)
8. Test solution volume:	200 mL (minimum)
9. Renewal of test solutions:	Minimum, at 48 h
10. Age of test organisms:	3 to 4 days post-hatch juveniles
11. No. organisms per test chamber:	Minimum, 5 for effluent and receiving water tests
12. No. replicate chambers per concentration:	Minimum, 5 for effluent tests and receiving water tests
13. No. organisms per concentration:	Minimum, 25 for effluent tests and receiving water tests
14. Feeding regime:	*Artemia* nauplii are made available while holding prior to the test; feed 0.2 mL of concentrated suspension of *Artemia* nauplii ≤24-h old, daily approximately 40 nauplii per mysid)

[1] Acute and chronic toxicity tests performed simultaneously to obtain acute/chronic ratios must use the same temperature and salinity.

TABLE 19. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR WEST COAST MYSID, *HOLMESIMYSIS COSTATA*, ACUTE TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (CONTINUED)

15. Test chamber cleaning:	Cleaning not required
16. Test solution aeration:	None, unless DO concentration falls below 4.0 mg/L; rate should not exceed 100 bubbles/min
17. Dilution water:	34 ± 2‰ salinity; Uncontaminated seawater (1 µm filtered) or hypersaline brine or equivalent (see Section 7, Dilution Water)
18. Test concentrations:	Effluents: Minimum of five effluent concentrations and a control Receiving Waters: 100% receiving water and a control
19. Dilution series:	Effluents: ≥0.5 dilution series Receiving Waters: None, or ≥0.5 dilution series
20. Endpoint:	Effluents: Mortality Receiving Waters: Mortality
21. Sampling and sample holding requirements:	Effluents: Grab or composite sample first used within 36 h of completion of the sampling period Receiving Waters: Grab or composite sample first used within 36 h of completion of the sampling period
22. Sample volume required:	1 L for effluents 2 L for receiving waters
23. Test acceptability criterion:	90% or greater survival in controls

SECTION 10

TEST DATA

10.1 BIOLOGICAL DATA

10.1.1 Death is the "effect" used for determining toxicity to aquatic organisms in acute toxicity tests.

10.1.2 Death is not as easily determined for some organisms. The criteria usually employed in establishing death are: (1) no movement of gills or appendages; and (2) no reaction to gentle prodding.

10.1.3 The death of some organisms, such as mysids and larval fish, is easily detected because of a change in appearance from transparent or translucent to opaque. General observations of appearance and behavior, such as erratic swimming, loss of reflex, discoloration, excessive mucus production, hyperventilation, opaque eyes, curved spine, hemorrhaging, molting, and cannibalism, should also be noted in the daily record.

10.1.4 The test chambers should be checked for early mortality during the first few hours of the test. The number of surviving organisms in each test chamber is recorded at the end of each 24-h period (Figure 4). When recognizable, dead organisms should be removed during each observation period.

10.1.5 The species, source, and age of the test organisms should be recorded.

10.2 CHEMICAL AND PHYSICAL DATA

10.2.1 In static tests, at a minimum, pH, salinity or conductivity, and total residual chlorine are measured in the highest concentration of test solution and in the dilution water at the beginning of the test, at test solution renewal, and at test termination. If total residual chlorine is not detected in effluent or dilution water at test initiation, it is unnecessary to measure total residual chlorine at test solution renewal or at test termination. It is also unnecessary to measure total residual chlorine in laboratory prepared synthetic dilution waters. DO, pH, and temperature are measured in the control and all test concentrations at the beginning of the test, daily thereafter, and at test termination.

10.2.1.1 It is recommended that total alkalinity and total hardness also be measured in the control and highest effluent concentration at the beginning of the test and at test solution renewal.

10.2.1.2 Total ammonia is measured in samples where toxicity may be contributed by unionized ammonia (where total ammonia might be $\geq$5 mg/L).

10.2.1.3 The DO should be monitored closely (every 2 h) for the first 4 to 8 h, to guard against rapid DO depletion, and is measured daily thereafter in all effluent concentrations in which there are surviving organisms, and at test termination. It is recommended that test solution DO be recorded continuously in the test chamber at the highest test solution concentration or in a surrogate vessel at a comparable test solution concentration and containing the standard complement of test organisms.

10.2.1.4 At a minimum, test solution temperature is measured at the beginning of the test, and daily thereafter. Temperature measurements are made by placing thermometers or other temperature sensing devices directly in test solutions or in a comparable volumes of water in chambers positioned in several locations among the test vessels to determine test solution temperatures. It is recommended that test solution temperature be recorded continuously in at least one test chamber or in a comparable volume of water in a surrogate vessel which is comparable to the test chambers.

10.2.2 In flow-through tests, at a minimum, pH, salinity or conductivity, total alkalinity, total hardness, and total residual chlorine are measured daily in the highest effluent concentration. DO and temperature are measured at the beginning of the test, daily thereafter in the control and all test concentrations, and at test termination.

10.2.3 The measurement of specific conductance is recommended because it is a very useful parameter in detecting transient fluctuations in the chemical characteristics of effluents, and will indicate errors in test dilutions.

10.2.4 Where acute toxicity test methods are utilized to determine permit limits for toxic chemicals, at a minimum, the concentration of the test material must be measured in each test concentration at test initiation, daily thereafter, and at test termination.

10.2.5 Methods used for chemical analysis should be those specified for Section 304(h) of the CWA (USEPA, 1993b). For salinity measurements, a refractometer may be used if calibrated with a sample of known salinity.

INDUSTRY/TOXICANT: _____
ADDRESS: _____
CONTACT: _____
EFFLUENT SERIAL NO: _____
NPDES PERMIT NO.: _____
SAMPLE COLLECTOR: _____

GRAB SAMPLE: COLLECTED (1) _____AM/PM; __/__/__(DATE)
(2) _____AM/PM; __/__/__(DATE)
(3) _____AM/PM; __/__/__(DATE)
(4) _____AM/PM; __/__/__(DATE)

COMPOSITE SAMPLE: COLLECTED (1) FROM: _____AM/PM; __/__/__(DATE) TO: _____AM/PM; __/__/__(DATE)
(2) FROM: _____AM/PM; __/__/__(DATE) TO: _____AM/PM; __/__/__(DATE)
(3) FROM: _____AM/PM; __/__/__(DATE) TO: _____AM/PM; __/__/__(DATE)
(4) FROM: _____AM/PM; __/__/__(DATE) TO: _____AM/PM; __/__/__(DATE)

PERSON CONDUCTING TEST: _____
TEST PERIOD:
BEGINNING: DATE _____ TIME _____
ENDING: DATE _____ TIME _____
TEST ORGANISM:
SPECIES: _____
AGE: _____
SOURCE: _____
DILUTION WATER USED:
DISSOLVED OXYGEN (mg/L) TOTAL RESIDUAL AMMONIA: _____
0 HRS _____ FREE AMMONIA: _____
24 " _____
48 " _____
72 " _____
96 " _____

Conc. or %	Test Container Number	Number of Live Organisms					Dissolved Oxygen (mg/l)					pH					Total Alkalinity (mg/l as $CaCO_3$)					Total Hardness (mg/l as $CaCO_3$)									
		0	24	48	72	96	0	24	48	72	96	0	24	48	72	96	0	24	48	72	96	0	24	48	72	96	0	24	48	72	96

Figure 4. Example of data sheet for effluent toxicity tests.

1. EXPOSURE CHAMBER

Total capacity: _____ mL

Test solution volume: _____ mL

Test solution surface area: _____ cm^2

Water depth (constant): _____ cm
(cyclic): _____ to _____ cm

2. FEEDING SCHEDULE

Not Fed: _____

Fed daily: _____

Fed irregularly:
(describe): _____

Food used: _____

3. AERATION

None: _____

Slow: _____ (Bubbles or mL/min)

Moderate: _____ " "

Vigorous: _____ " "

From: _____ AM/PM; ___/___/___ (DATE)
To: _____ AM/PM; ___/___/___ (DATE)

4. SCREENED ANIMAL ENCLOSURES

Not used: _____

Used: _____
(cm) Diameter

5. Condition/appearance of surviving organisms at end of test: (i.e., alive but immobile; loss of orientation; erratic movement; etc.) _____

_____

_____

6. Comments: _____

_____

_____

NPDES NO: _____ Inspection Date: _____ Outfall number: _____
Facility Name: _____ Test Date: _____ Macro Test: _____
City Name: _____ Inspection Code: _____ Type Macro: _____
County Name: _____ Type Inspection: _____ Expo Time: _____
Receiving Water: _____ Date Info to WSD: _____ Results: _____
Permit Issued: _____ Date Info to State: _____ Fish Test: _____
Permit Expires: _____ Date of WMD Action: _____ Type Fish: _____
SIC Code: _____ Data of Static Action: _____ Expo Time: _____
Present Treatment: _____ Type of Action: _____ Results: _____
Remarks: _____ Annual Status Update: _____ Remarks: _____

Figure 5. Check list on back of effluent toxicity data sheet.